CDKN2A Germline Mutations in U.K. Patients with Familial Melanoma and Multiple Primary Melanomas

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We report six of 16 U.K. melanoma families and two of 17 patients with multiple primary melanomas and a negative family history who have between them four different functionally damaging mutations of the CDKN2A (p16) gene: an Arg 24 Pro substitution in exon 1 in one family, a stop codon at codon 44 of exon 1 in one family, and a Met 53 Ile substitution in exon 2 in four families. One multiple primary melanoma patient also has the Met 53 Ile mutation and a second has a G-T substitution at the IVS2 + 1 splice donor site. Our data together with other recent publications from France and the U.S.A. indicate that screening melanoma kindreds with only two affected family members for CDKN2A mutations is justified. ***Key words: familial melanoma/growth suppressor/p16/tumor suppressor. J Invest Dermatol 111:269–272, 1998***

Genetic linkage analysis has shown that a proportion of patients with both familial and sporadic cutaneous melanoma show loss of heterozygosity on chromosome 9p21 (Cannon-Albright *et al*, 1992, 1994). Studies of familial melanoma patients in the U.S.A. (Hussussian *et al*, 1994; Kamb *et al*, 1994; Fitzgerald *et al*, 1996), Australia (Walker *et al*, 1995; Holland *et al*, 1995), The Netherlands (Gruis *et al*, 1995) Sweden (Borg *et al*, 1996; Platz *et al*, 1997), England (Harland *et al*, 1997), and France (Soufir *et al*, 1998) have detected mutations of the *CDKN2A* gene (also known as p16 or MTS1 gene) in approximately one-sixth of cases of familial melanoma. *CDKN2A* is a small gene consisting of three exons and is situated on chromosome 9p21. The normal function of CDKN2A is to encode the *CDKN2A* protein that inhibits the cyclin dependent kinases *CDK4* and *CDK6*. These kinases are responsible for controlling the passage of cells through the G1 checkpoint by inhibiting the phosphorylation of the retinoblastoma protein (Serrano *et al*, 1995). Wild-type *CDKN2A* is therefore regarded as a tumor suppressor gene, and mutations have been detected in a wide range of human malignancies, including melanoma (Foulkes *et al*, 1997).

We report results of screening 16 families drawn from a population based melanoma register in the U.K. We have identified three distinct CDKN2A mutations in six families, and two mutations in 17 patients with multiple primary melanomas and no evidence of a family history of melanoma.

MATERIALS AND METHODS

Patients Melanoma patients were identified through the records of the Scottish Melanoma Group, a population based registry of all patients in whom invasive cutaneous melanoma is diagnosed in Scotland. At present there are over 8000 melanoma patients registered (MacKie *et al*, 1997), and the families identified for this study were selected on the basis of being located in the west of Scotland and being willing to attend together with unaffected family members who would all undergo full skin examination and donate a blood sample for DNA extraction. All patients had at least one other living family member with pathologically confirmed invasive melanoma who also donated DNA.

Details of the families are shown in **Table I** and the family trees of those with mutations detected are shown in **Fig 1**. The largest family, mel 2, has to date four family members with invasive melanoma and the index case in this family has to date four distinct primary cutaneous melanomas. His brother, sister, and son all have one invasive primary melanoma, and his second son who also carries the mutation has a medium sized congenital naevus on the dorsum of his right foot. One other family has three affected members in three generations and the remaining four all have two affected family members.

The 17 patients screened with multiple primary melanomas are representative with regard to sex distribution, age at presentation, and thickness of first primary melanoma of a series of 108 patients with multiple invasive primary melanomas in which we have investigated risk factors and prognostic factors in individuals who have multiple primary melanomas Burden *et al*, 1994). The 17 who gave DNA for this study comprised eight males and nine females with a mean age at presentation with first primary of 49 y (range 22–70 y). Fourteen had two invasive primary melanomas, two had three, and one had eight primary melanomas. These patients do not have other nonmelanoma malignancies.

Design and synthesis of oligonucleotide primers The polymerase chain reaction primer sequences used to amplify exons 1 and 2 of *CDNK2A* were designed with the aid of the OLIGO Version 3.4 program (Wojciech Rychlik; MEDPROBE). The sequences of the primers for exon 3 were identified from Kamb *et al* (1994) and synthesized.

The polymerase chain reaction and single stranded conformational polymorphism analysis Standard conditions were used for polymerase chain reactions. For single stranded conformational polymorphism analysis (Orita *et al*, 1989), 4 μl polymerase chain reaction product was mixed with 6 μl of single stranded conformational polymorphism loading buffer. Samples were denatured at 95°C for 5 min then chilled on ice and 4 μl loaded per well. Products were resolved by running in 0.5% mutation detection enhancement gels (Flowgen, Lichfield, Staffs, U.K.) and 5% glycerol at 4 W for 16 h at room temperature. The DNA bands were visualized by silver staining then photographed.

DNA sequencing Double stranded polymerase chain reactions were carried out on the relevant DNA, and the DNA products purified by ethanol precipitation and dissolved in 20 μl of sterile double distilled water. Sequencing reactions were then carried out using the Thermo Sequenase Dye Terminator Cycle sequencing premix kit (Amersham, Essex, U.K.)

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Table I. Details of 16 U.K. melanoma families screened for germline mutations of the CDKN2A gene[a]

Case no.	Index case and age at diagnosis of first primary	Affected relatives	Mutation detected
1	Female (39) (2 primary melanomas)	Mother and grandmother	Exon 2 Met 53 Ile
2	Male (31) (4 primary melanomas)	Brother, sister, and son	Exon 1 Arg 24 Pro
3	Female (33) (2 primary melanomas)	Mother (55)	Exon 1 tyr 44 stop
4	Male (46) (2 primary melanomas)	Sister (46) (3 primaries)	Exon 2 Met 53 Ile
5	Female (27) (2 primary melanomas)	Cousin (36)	Exon 2 Met 53 Ile
6	Female (34)	Daughter (21)	Exon 2 Met 53 Ile
7	Female (45)	Brother (43)	–
8	Female (41)	Sister (23)	–
9	Male (21)	Mother (56)	–
10	Male (53)	Sister (45)	–
11	Male (63)	Daughter (32)	–
12	Female (37)	Father (69)	500 G/C
13	Male (34)	Brother (36)	–
14	Female (31)	Mother (68)	–
15	Female (61)	Son (48)	–
16	Female (16)	Father	–
Patients with multiple primary melanomas and CDKN2A mutations			
Male (28)	8 primaries (4 synchronous)		Exon 2 Met 53 Ile
Female (29)	2 primaries (synchronous)		IVS2 + 1 G > T

[a]All melanomas included in this table are invasive, i.e., level 2 or deeper.

using standard protocols for the ABI 373 sequencer. Data are presented in chromatogram form where C = blue, A = green, T = red, and G = black. Heterozygosity is generally represented by two superimposed peaks and designated N (**Fig 2**).

RESULTS

All three exons of the *CDKN2A* gene were scanned by single stranded conformational polymorphism analysis for mutations, and all variant bands subsequently analyzed by direct sequencing to confirm the presence of a mutation. Three distinct mutations were detected in six of the 16 families screened, and a seventh family had a previously recognized polymorphism (Chaubert *et al*, 1996). We did not detect any CDKN2A abnormality in the remaining nine families.

The three mutations detected were a substitution of proline for arginine in codon 24 of exon 2 in the largest family studied, which to date has four affected family members (UKFM 2), a stop codon at 44 in exon 1 in a mother and daughter (UKFM 3), and an isoleucine substitution for methionine at codon 53 of exon 2 in four other families comprising a brother and sister, two cousins, and two mothers and daughters (UKFM 1, 4, 5, and 6) The one family that showed the 500 G/C polymorphism consisted of a father and daughter (UKFM 12).

Two of the 17 patients with multiple primary melanomas and a negative family history had CDKN2A mutations. One, a male who has to date developed eight separate invasive primary melanomas and who has no detectable defect in DNA repair, has the exon 2 Met 53 Ile mutation. The second is a female who had two synchronous primary melanomas excised 12 y ago and has a G-T substitution at the IVS2 + 1 splice donor site. As G is the first base of the intron this will affect splicing as the first two bases always have a consensus sequence GT. Seven of the other patients screened with multiple primary melanomas showed the G-C polymorphism 29 bp downstream of the coding region of exon 3, and the remaining eight showed neither mutations nor polymorphisms.

All of the mutations detected in this series of patients are likely to be functionally deleterious. The G_{47} to C transversion in codon 24 (CGG to C**C**G) in family UKFM 2 is a missense mutation that changes the basic amino acid arginine to proline ($Arg_{24}Pro$), which is neutral and hydrophobic and would therefore be expected to have both a structural and a functional effect on the protein. Harland *et al* has recently shown that this variant is defective in binding to CDK4 but normal in binding to CDK6, and in addition has shown no CDK binding for the Met 53 Ile mutation (Harland *et al*, 1997). As a premature termination codon, the tyr 44 stop codon in exon 1 seen in family UKFM 3 is highly likely to be functionally deleterious.

DISCUSSION

To date, studies on melanoma families worldwide suggest that the presence and frequency of *CDKN2A* mutations in familial melanoma varies between continents, and also between patient groups in the same continent. For example, Hussussian *et al* (1994) reported on 13 of 18 families from the U.S.A. with *CDKN2A* mutations (72%), whereas Kamb *et al* (1994), also in the U.S.A., found mutations in only two of 36 families; however, the US study most relevant to this study is that of Fitzgerald *et al* (1996), who report five CDKN2A mutations in 33 patients from 28 families in a clinic based population. It is of importance that these families were defined, as were ours, as having only one affected first degree family member, a population much commoner than the larger cancer families studied by family cancer geneticists. The recently published french study of 48 families (Soufir *et al*, 1998) further emphasizes the fact that CDKN2A mutations are found in kindreds with only two affected family members (eight of 28 or 28%) as well as in kindreds with larger numbers of affected members. In other parts of Europe, *CDKN2A* mutations have been recorded in a large Dutch kindred (Gruis *et al*, 1995) and in two of 10 and five of 64 Swedish families, respectively (Borg *et al*, 1996; Platz *et al*, 1997), and in six of 27 U.K. families (MacGeoch *et al*, 1994; Harland *et al*, 1997). In Australia, Walker *et al* (1995) found *CDKN2A* mutations in seven of 18 Australian melanoma pedigrees (39%), whereas a second Australian study (Holland *et al*, 1995) showed only one of 17 melanoma families to express *CDKN2A* mutations. The Met 53Ile substitution found in four of our families was first reported by Walker *et al* (1995), and results from a G-C substitution at codon 153, and was found by Walker to segregate with the putative melanoma chromosome in a large Australian kindred. They stated that this Australian family "probably originated

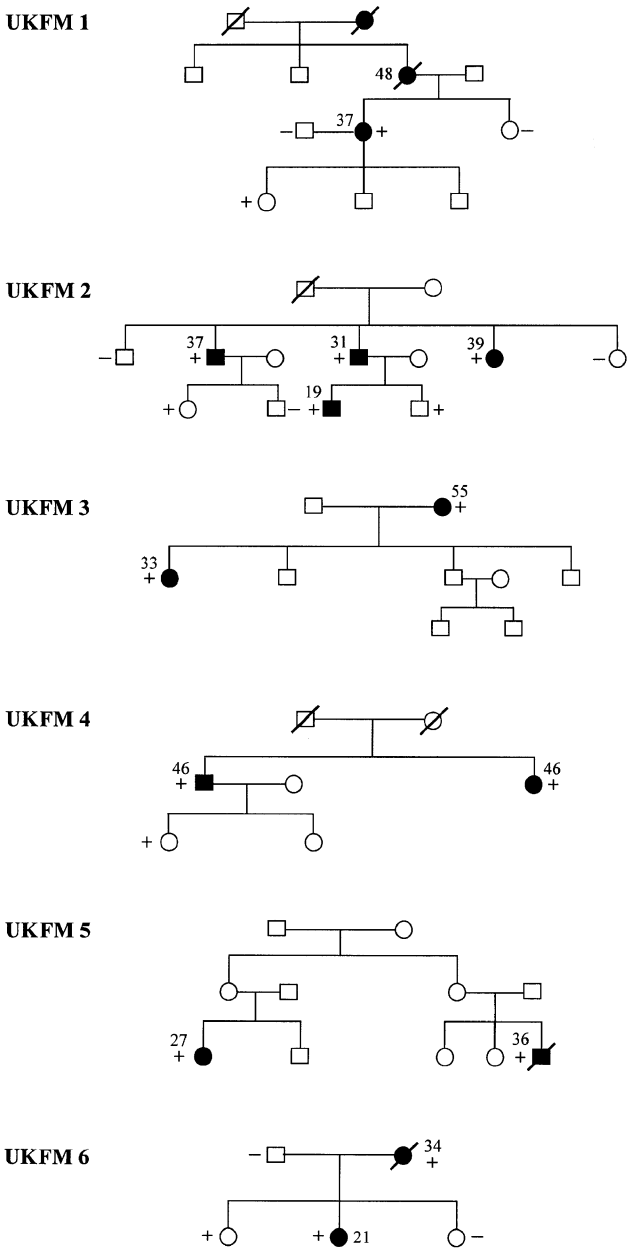

Figure 1. Family trees of the six families in which CDKN2A mutations were detected. Filled squares and circles indicate males and females with invasive melanoma, and figures adjacent to these symbols indicate age at diagnosis of first invasive primary melanoma. A plus sign indicates the presence of a mutation detected by sequencing. In families UKFM 1, 4, 5, and 6 this is the Met 53 Ile mutation in exon 2, in UKFM 2 this is Arg 24 Pro in exon 1, and in UKFM 3 this is a stop codon at codon 44 of exon 1.

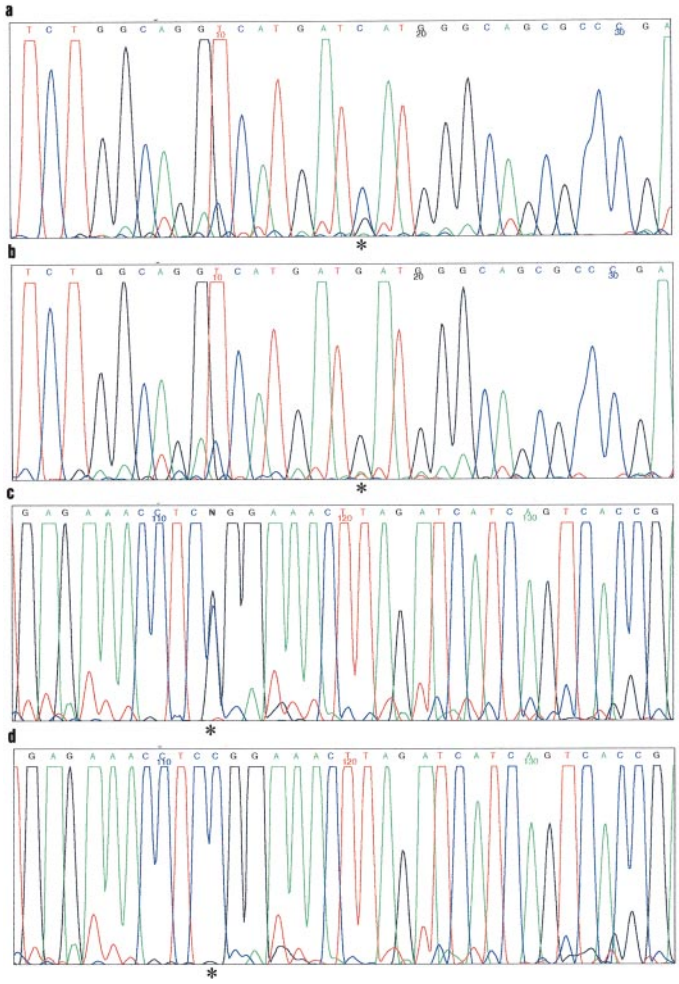

Figure 2. Chromatograms showing the mutations detected. (*a*) The Met 53 Ile substitution detected in families mel 1, 4, 5, and 6; (*b*) the normal chromatogram picture for the relevant section of the gene. (*c*) The G-C polymorphism at base 500 of the three prime untranslated region of exon 3, and (*d*) the more commonly seen pattern in this area. In all chromatograms the relevant area is marked with an asterisk.

from Scotland." Collaborative studies are currently in progress between ourselves and the Australian group to establish whether or not the families share common ancestry.

The tyr 44 stop codon in exon 1 of CDKN2 has previously been reported in non-small cell lung cancer (Washimi *et al*, 1995) but this appears to be the first report of this particular mutation in melanoma.

In our family UKFM 2 it appears that in generation 2 among the five siblings, the *CDKN2A* mutation segregates with the disease, and surveillance continues of the two children in generation 3 who have the mutation but not as yet melanoma. One of these children has an intermediate sized congenital naevus. Congenital naevi have to date not been reported in patients with CDKN2A mutations, and there is controversy about their malignant potential. In other reports, clear segregation of melanoma with the gene mutation is not invariably the case, although in other pedigrees there appears in some cases to be linkage to chromosome 9, but no *CDKN2A* mutation. This could be due to another as yet unidentified suppressor gene in this area of chromosome 9, or to inappropriate assignment to 9p21, based on a weak LOD score.

The genetics of patients with multiple primary melanomas but no family history have not as yet been reported in detail. Brill *et al* (1997) reports five of 34 patients with two or more primary melanomas with CDKN2A mutations, but subsequently found two of the five to have a positive family history. They conclude that the presence of such mutations may indicate an occult family history of melanoma. Harland *et al* (1997) record no mutations in three patients studied with multiple primary melanomas. Our sample of 17 patients with multiple primary melanomas, two of whom have CDKN2A mutations, indicates that this group of patients merits further study.

In conclusion, this study has added six new families to the known population of families with melanoma and CDKN2A mutations. Our data together with that of Fitzgerald and Soufir suggest that investigating families with only two affected members is worthwhile. Such families will be found in the care of dermatologists, oncologists, or surgeons and family cancer services may well not be notified.

In future, knowledge of CDKN2A status may well be relevant to melanoma screening, surveillance, or early detection activities

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